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(54) Title: COMBINATION THERAPIES USING VITAMIN B12 AND THERAPEUTIC AGENTS FOR TREATMENT OF VIRAL, PROLIFERATIVE AND INFLAMMATORY DISEASES

(57) Abstract: Pharmaceutical compositions for treating viral, proliferative and inflammatory diseases are disclosed comprising an amount of pharmaceutically acceptable vitamin B12 compounds in combination with anti-viral, anti-proliferative and anti-inflammatory compounds. Vitamin B12 compounds are administered separately, simultaneously or in combination with anti-viral, anti-proliferative and/or anti-inflammatory compounds to provide an enhanced therapeutic effect for treating viral, proliferative and inflammatory diseases.

**Combination therapies using Vitamin B12 and Therapeutic Agents for Treatment of  
Viral, Proliferative and Inflammatory Diseases**

**5 Field of the Invention**

The present invention provides pharmaceutical compositions and methods of use for the treatment of viral, inflammatory and/or proliferative diseases with a vitamin B12 compound in combination with an anti-viral, anti-inflammatory and/or inflammatory compounds.

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**Background of the Invention**

**Review of viral, inflammatory and proliferative diseases**

15 Diseases and disorders that have significant inflammatory, viral and/or proliferative components are widespread and affect millions of people worldwide. Selected examples of inflammatory, viral and/or proliferative diseases include multiple sclerosis, diabetes, restenosis, cancer, hepatitis C, HIV/AIDS and genital warts. These types of diseases share common disease processes and as a result often share or possess related/common therapies.

20

***Viral Diseases***

Viruses are potent infectious pathogenic agents that cause important functional alterations of the invaded cells, often resulting in cellular death. It is generally acknowledged that the cell  
25 injury in viral diseases includes not only direct damages inflicted by the proliferation of viruses but also various immunologic reactions elicited by infection with viruses. In fact, the consequences of a viral disease depend upon several viral and host factors such as the quantity of infecting viral particles, the speed of viral multiplication and spread, the impact on cell functions, the host's secondary responses to the cellular injury, and both the immunologic and  
30 the non-specific defenses of the host. In general, the effects of a viral disease include asymptomatic infections, both acute and chronic clinical diseases and induction of various types of cancers. One example of a well known viral disease is the viral hepatitis infection which results in chronic or acute inflammation of the liver and can lead to hepatocellular carcinoma in some cases. To date, there are several types of hepatitis viruses that have been

identified, including hepatitis viruses A, B, C, D E and G that are prevalent among the population. For instance, it has been estimated by the Liver Foundation International that there are more than 520 million individuals worldwide that suffer from either hepatitis B or hepatitis C.

5

### ***Inflammatory Diseases***

Inflammatory diseases are a diverse set of disorders that are characterized by the influx of certain cell types and mediators, the presence of which can lead to tissue damage and sometimes death. Essentially, the inflammatory cascade is a complex process that involves the triggering of immunological response, the release of chemokines, cytokines and toxic agents by the activated cells, the up-regulation of cell surface adhesion molecules and trans-endothelial cell migration. Typically, inflammation occurs as a defensive response to invasion of the host by foreign material, bacteria or to mechanical trauma, toxins and neoplasia. Autoimmune responses by intrinsic stimulation also can induce inflammatory responses.

15

One example of inflammatory disease is multiple sclerosis ("MS"). MS is a multi-factorial inflammatory disease of the human central nervous system resulting in the slowing of electrical conduction along the nerve. It is estimated that close to a third of a million people in the United States have MS. MS is believed to result from inflammation and breakdown in the myelin surrounding the nerve fibers of the central nervous system. The disease is characterized by an increase in the infiltration of inflammatory cells, loss of oligodendrocytes, and increased gliosis (astrocyte hypertrophy and proliferation). (For review see Amit et al., 1999; Pouly et al., 1999; Steinman et al., 1993; Miller, 1994).

25

### ***Proliferative diseases***

Cancer is the most well known proliferative disease. Specifically, cancer is a generic term representing a collection of diseases arising from mutations of key molecules that regulate cell proliferation, invasion, and metastasis. The ability of tumor cells to metastasize involves deregulation via overproduction or mutation of genes that allow cells to invade out of the tissue of origin, survive in a contact-independent manner, escape immune recognition, lodge at a distant site, then invade to a suitable place within the new tissue and grow there. The molecules that are commonly involved in tumor initiation, progression and metastasis include adhesion molecules, growth factor receptors, factors regulating the cytoskeleton, master

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switches regulating cell cycle, proliferation repressor genes, proteases and transcription factors. However, the ability of most tumors to kill is directly related to their capacity to invade and ultimately to metastasize.

5 ***Viral, inflammatory and proliferative diseases share common disease processes***

There are several classes of molecules and disease processes that are common to all these diseases. These include elevated expression of adhesion molecules, cytokines and matrix metalloproteinases, increased cell proliferation and migration, increased inflammatory cell activation and infiltration, increased angiogenesis, and increased tissue destruction and  
10 dysfunctional matrix remodeling. Consequently, compounds for the treatment of these diseases are aimed at altering the immune system, cell proliferation, cell adhesion and migration, cytokine levels or activities or viral replication.

***Need for enhancing compounds to decrease side effects***

15 Although many anti-inflammatory, anti-viral and anti-proliferative compounds are effective at treating inflammatory, viral and proliferative diseases, these compounds often cause side effects that are exacerbated when the dose is increased. Side effects can include local injection reactions, flu-like syndrome and depression. Accordingly, some patients are unable to tolerate the doses needed to achieve a therapeutic effect.

20

Interferon is an example of a common anti-inflammatory, anti-viral, *and* anti-proliferative therapeutic compound with many undesirable side effects, which are exacerbated at high doses. Accordingly, some patients are unable to tolerate the doses needed to achieve a therapeutic effect. In addition, although many anti-viral, anti-inflammatory or anti-  
25 proliferative compounds may have shown promise *in vitro* against inflammatory, viral, or proliferative diseases, very high doses may be required *in vivo*; such doses often being toxic or causing severe side effects. Therefore, the limitation of these therapies may be in using sufficient levels of the compound to achieve maximal efficacy in the absence of side effects.

30 Thus, compounds that can be combined with anti-viral, anti-proliferative or anti-inflammatory compounds to increase effectiveness of treatments for inflammatory, viral or proliferative diseases are necessary. In addition, compounds that can reduce the doses and/or reduce frequency of administration to reduce side effects and to maintain or improve efficacy are necessary.

For example, a compound that can improve efficacy of interferon-beta in the treatment of MS would potentially reduce the amount and/or frequency of interferon-beta administration, reducing side effects induced by interferon-beta and possibly reducing the occurrence of neutralizing antibodies to interferon-beta in MS patients.

Neutralizing antibodies to interferon-beta occur in about one third of MS patients treated with interferon-beta and are positively correlated with a loss of therapeutic efficacy of interferon-beta (Deisenhammer et al., 1999).

In addition, these compounds would not only enhance the efficacy in current or potential anti-viral, anti-proliferative or anti-inflammatory therapies but would also broaden use of these therapies into many other inflammatory, proliferative and viral diseases.

There have been reports of anti-inflammatory agents being tested in combination for treatment of inflammatory diseases such as multiple sclerosis. However, these combination therapies have resulted in limited efficacy or no reduction in side effects. For example, combination therapy comprising copaxone and interferon-alpha did not improve clinical scores in EAE-treated mouse (Brod et al., 2000). While novantrone, an anti-proliferative drug, is currently tested in clinical trials for combination therapy with interferon-beta, novantrone has a significant side effect profile including serious cardiac toxicity. Novantrone is restricted in its use because the risk of heart disease increases with the cumulative dose. According to the Food and Drug Administration, patients with MS should ordinarily not receive more than 8 to 12 doses of novantrone, administered over two to three years.

Accordingly, there is a need to develop therapies for viral, inflammatory or proliferative diseases that result in sufficient levels of anti-viral, anti-inflammatory and anti-proliferative compounds to achieve maximal efficacy with minimal side effects. There is a need to develop anti-viral, anti-inflammatory and anti-proliferative combination therapies for the treatment of viral, inflammatory or proliferative diseases wherein the combined elements have an enhancing therapeutic effect, while minimizing side effects.

***Vitamin B12***

Vitamin B12 is a cobalt-containing B complex vitamin that has various effects on biological processes *in vivo*. Vitamin B12 has a well-elucidated family of analogues and conjugates.

5 Vitamin B12 compounds have been known to be involved in metabolic processes; and deficiency of vitamin B12 has been known to provoke pernicious anemia and neurological disorders. It is a co-factor essential in the metabolic pathway leading to synthesis of DNA, cell division, as well as cellular metabolism. Biochemical evidence suggests that vitamin B12 compounds may up-regulate gene transcription and thereby protein synthesis (Watanabe et al,

10 1994). It has been also suggested that vitamin B12 compounds play an important role in immune system regulation (Tamura et al, 1999, Sakane et al, 1982).

Specifically, vitamin B12 compounds have been suggested to possess some or limited anti-viral (Weinberg et al, 1995, 1998, Lott et al, 2001, Poydock, 1979, Tsao et al., 1990), anti-proliferative (Nishizawa et al, 1997, Shimizu, 1987, Poydock et al., 1979, 1985) and anti-inflammatory activities (US Patent # 5,508,271, US Patent # 5,964,224, US Patent # 5,716,941) on their own. Vitamin B12 has also been tried in combination with other therapeutic agents (EP Patent # 0835660, US Patent # 6,096,737) for the treatment of specific inflammatory diseases or proliferative diseases. However, to date, there has been no scientific

15 evidence demonstrating an enhanced therapeutic effect of vitamin B12 in combination with anti-viral, anti-inflammatory or anti-proliferative agents for treatment of viral, inflammatory or proliferative disease.

It is therefore an object of the present invention to provide a combination therapy using vitamin B12 compounds with anti-viral, anti-inflammatory or anti-proliferative agents for the treatment of viral, inflammatory or proliferative diseases. Interferon, a multi-faceted molecule that possesses anti-inflammatory, anti-proliferative and anti-viral activities, is an example of the combination therapy with vitamin B12. The invention is also supported by usage of other anti-viral, anti-inflammatory and anti-proliferative compounds such as taxol

25 and P-16 with vitamin B12 compounds.

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The invention comprises a pharmaceutical composition for the treatment of a disease selected from a group of viral diseases; proliferative diseases; inflammatory diseases; proliferative and  
5 inflammatory diseases; proliferative and viral diseases; viral and inflammatory diseases; and proliferative, viral and inflammatory diseases; comprising: (1) at least one vitamin B12 compound; and (2) at least one second compound selected from the group consisting of: anti-viral compound; anti-proliferative compound; and anti-inflammatory compound.

10 Another embodiment of this invention comprises a pharmaceutical composition for the treatment of proliferative diseases, such as cancer or astrocytoma or glioma comprising at least one vitamin B12 compound and at least one anti-proliferative compound such as paclitaxel, or at least one vitamin B12 compound and at least one interferon compound.

15 Yet another embodiment of this invention comprises a pharmaceutical composition for the treatment of viral diseases, such as hepatitis B, hepatitis C, herpes, or vesicular stomatitis comprising at least one vitamin B12 compound and at least one anti-viral compound, or at least one vitamin B12 compound and at least one interferon compound.

20 Yet another embodiment of this invention comprises a pharmaceutical composition for the treatment of inflammatory diseases comprising at least one vitamin B12 compound and at least one anti-inflammatory compound, or at least one vitamin B12 compound and at least one interferon compound.

25 Another embodiment of this invention comprises a pharmaceutical composition for the treatment of MS comprising at least one vitamin B12 compound and at least one interferon compound, such as interferon-beta, or at least one vitamin B12 compound and paclitaxel.

Another embodiment of this invention comprises a pharmaceutical composition for the  
30 treatment of MS comprising at least one vitamin B12 compound and a peptide selected from the group consisting of P16, S-3, S-7, P-32, V-2 and V-3.

Another aspect of this invention is a pharmaceutical composition for the treatment of hepatitis B comprising vitamin B12 and interferon-alpha or interferon-beta.

Another aspect of this invention is a pharmaceutical composition for the treatment of hepatitis C comprising vitamin B12 and interferon-alpha or interferon-beta.

- 5 Another aspect of this invention is a pharmaceutical composition according to any of the aspects outlined above wherein the vitamin B12 compound is conjugated to the second compound.

- 10 Another aspect of this invention is a method of treating a viral, proliferative or inflammatory disease, including MS, hepatitis B and hepatitis C, comprising the step of administering to a patient any of the pharmaceutical compositions outlined above.

- 15 Another aspect of this invention is a method of treating a viral, proliferative, or inflammatory disease, including MS, hepatitis B and hepatitis C, comprising the steps of administering the vitamin B12 compound at a frequency selected from the group consisting of: more than once daily, daily, more than once weekly, weekly, more than once monthly and monthly, and administering the second compound at a frequency selected from the group consisting of: more than once daily, daily, more than once weekly, weekly, more than once monthly, and monthly.

- 20 An other aspect of this invention is a method of treating a viral, proliferative, or inflammatory disease, including MS, hepatitis B and hepatitis C, comprising the steps of administering to a patient, either together or separately, one or more vitamin B12 compound, and one or more anti-inflammatory, anti-viral or anti-proliferative compound.

- 25 Encompassed within this invention are any of the above treatments wherein the dose of vitamin B12 compound is between 10 and 2500 mg, and/or the dose of anti-inflammatory, anti-viral or anti-proliferative compound is similar or less than the therapeutic dose range used for the anti-inflammatory, anti-viral or anti-proliferative compound.

- 30 Another aspect of this invention is the use of any of the compounds described above to treat a viral, proliferative, or inflammatory disease.



**Definitions**

Prior to setting forth the invention, it may be helpful to an understanding thereof to first set forth definitions of certain terms that will be used hereinafter.

5

"Anti-inflammatory compounds" means a class of compounds for treating inflammatory diseases. The compounds include compounds in research, in development and compounds marketed and sold. This class of compounds includes aspirin, sodium salicylate, choline salicylate, salicylsalicylic acid, diflunisal, salsalate, indomethacin, sulindac, phenylbutazone, oxyphenbutazone, tolmetin, ibuprofen, feropfen, flurbiprofen, ketoprofen, mefanamic acid, meclofenamate, piroxicam, naproxen, hydrocortisone, prednisolone, 6-alpha-methylprednisolone, triamcinolone, dexamethasone, betamethasone, cyclosporine, mycophenolate mofetil, cyclophosphamide, antisense ICAM-1, 6-mercaptopurine, tacrolimus, muromonab-CD3, ISA247, alefacept, efalizumab, infliximab, azathioprine, methotrexate, sulfasalazine, CT-3<sup>TM</sup>, COX-2 inhibitors, OMS-103HP<sup>TM</sup>, CAB-2<sup>TM</sup>, LDP-01<sup>TM</sup>, IPL550,260<sup>TM</sup>, IPL512,602<sup>TM</sup>, Reliflex<sup>TM</sup>, LFA-1 antagonist, IC74, interferon beta, Avonex<sup>TM</sup>, Betaseron<sup>TM</sup>, Betaferon<sup>TM</sup> or Rebif<sup>TM</sup>, analogues of Avonex<sup>TM</sup>, Betaseron<sup>TM</sup>, Betaferon<sup>TM</sup> or Rebif<sup>TM</sup>, interferon compounds, taxanes, Taxol<sup>TM</sup>, microtubule stabilizing agents, analogues of microtubule stabilizing agents, glatiramer acetate, analogues of glatiramer acetate, Novantrone<sup>TM</sup>, Antergren<sup>TM</sup>, Campath<sup>TM</sup>, Adapalene<sup>TM</sup>, nitric oxide synthase inhibitors, anti-TNF or IL-1 compounds and antagonists, antibodies to CD52, retinoic acid antagonists, diacerhein, diacerhein analogues, adhesion peptides, MAF peptides, cytokines, hyaluronic acid (HA) binding peptides, RHAMM peptides, integrins, and mixtures thereof. The singular form, "anti-inflammatory compound", may mean any one or more compounds from the class of anti-inflammatory compounds.

"Anti-proliferative compounds" means a class of compounds for treating proliferative diseases. The compounds include compounds in research, in development and compounds marketed and sold. This class of compounds includes but not limited to: altretamine (hexamethylmelamine, Hexalen), anastrozole (Arimidex), Exemestane (Aromasin), bicalutamide (Casodex), busulfan (Myleran), capecitabine (Xeloda), chlorambucil (Leukeran), cyclophosphamide (Cytosan), diethylstilbestrol diphosphate (Stilphostrol), estramustine (Emcyt), etoposide (VP-16, Vepesid), flutamide (Eulexin), hydroxyurea (Droxia), Hydrea, Mylocel, letrozole (Femara), leucovorin calcium (Leucovorin), levamisole (Ergamisol), lomustine (CCNU, CeeNU), megestrol (Megace), melphalan (Alkeran), mercaptopurine (6-

MP, Purinethol), methotrexate (Methotrexate, Rheumatrex), mitotane (Lysodren), nilutamide (Nilandron), procarbazine (Matulane), tamoxifen (Nolvadex), testolactone (Teslac), thioguanine, tretinoin (Vesanoid), mechlorethamine, 5-fluorouracil, cytarabine, gemcitabine, vinblastine, vincristine, vinorelbine, paclitaxel, etoposide, irinotecan, topotecan; leuprolide, flutamide, doxorubicin, bleomycin, epirubicin, mitomycin, interferon compounds, carmustine, lomustine, cisplatin, mitoxantrone (Novantrone). The singular form, "anti-proliferative compound", may mean any one or more compounds from the class of anti-proliferative compounds.

10 "Anti-viral compounds" means a class of compounds which includes any therapeutic compounds for treating viral diseases. The compounds include compounds in research, in development and compounds marketed and sold. The class of anti-viral compounds includes interferon compounds, acyclovir, adefovir, abacavir, amprenavir, didanosine, fomivirsen sodium, dipivoxil, adenine, arabinoside, famciclovir, ganciclovir, lopinavir, ritonavir, lamivudine, nelfinavir mesylate, stavudine, trizivir, amivudine, lobucavir, zidovudine, indinavir, nevirapine, delavirdine, saquinavir, efavirenz, ribavirin, foscarnet, n-docosanol, oseltamivir, valacyclovir, palivizumab, doxuridine, miquimod, vidarabine, trifuridine, ritonavir, neuraminidase inhibitor, tenofovir, disoproxil fumarate and zalcitabine. The singular form, "anti-viral compound", may mean any one or more compounds from the class of anti-proliferative compounds.

"CFA" means complete Freund's adjuvant.

25 "DM20" means an isoprotein proteolipid protein. It is a major integral membrane protein of the central nervous system (CNS). DM20 is normally expressed in early (post-natal) stages of growth.

"EAE" or "experimental autoimmune encephalomyelitis" means a mouse model, specifically, the immunosuppressive mouse model for multiple sclerosis.

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"enhanced", "enhancing" or "enhances", used in the context of, for example, "enhanced effectiveness", means an enhanced therapeutic effect, and includes a synergistic effect.

"Inflammatory diseases" means a class of diverse diseases and disorders that are characterized by any one of the following: the triggering of an inflammatory response; an upregulation of any member of the inflammatory cascade; the downregulation of any member of the inflammatory cascade. Inflammatory diseases include diabetes, arteriosclerosis, inflammatory aortic aneurysm, restenosis, ischemia/reperfusion injury, glomerulonephritis, 5 sacoidosis cancer, restenosis, reperfusion injury, rheumatic fever, systemic lupus erythematosus, rheumatoid arthritis, Reiter's syndrome, psoriatic arthritis, ankylosing spondylitis, coxarthrititis, inflammatory bowel disease, ulcerative colitis, Crohn's disease, pelvic inflammatory disease, multiple sclerosis, diabetes, osteomyelitis, adhesive capsulitis, oligoarthritis, osteoarthritis, periartthritis, polyarthrititis, psoriasis, Still's disease, synovitis, 10 Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, osteoporosis, inflammatory dermatosis and wound healing. The singular term "inflammatory disease" includes any one or more diseases selected from the class of inflammatory diseases, and includes any compound or complex disease state wherein a component of the disease state includes a disease selected from the class of inflammatory diseases. 15

"Interferon compounds" means interferon-alpha, interferon-alpha analogues, interferon-alpha derivatives, interferon-alpha conjugates, interferon beta, interferon-beta analogues, interferon-beta derivatives, interferon-beta conjugates and mixtures thereof. Interferon-alpha and 20 interferon-beta genes may be altered by, for example, oligonucleotide directed mutagenesis to produce interferon-beta analogues thereof, such as the human recombinant cysteine depleted or cysteine replaced analogues. Further, identity or location of more than one amino acid may be changed by targeted mutagenesis. The primary amino acid sequence of the protein may be augmented by glycosylation or by other supplementary molecules such as lipids, phosphate, 25 and acetyl groups. Further, individual amino acid residues in the chain may be modified by oxidation, reduction, or other derivatization. The interferon-alpha or interferon-beta protein may be cleaved to obtain the fragments which retain activity. The whole protein or its fragments can be fused with other peptides and proteins such as immunoglobulins and other cytokines. Interferon-alpha and interferon-beta conjugates may represent, for example, a 30 composition comprising interferon-beta coupled to a non-naturally occurring polymer comprising a polyalkylene glycol moiety. Preferred interferon compounds include Roferon®, Intron®, Alferon®, Infergen®, Omniferon®, Alfacon-1, interferon-alpha, interferon-alpha analogues, pegylated interferon-alpha, polymerized interferon-alpha, dimerized interferon-alpha, interferon-alpha conjugated to carriers, interferon-alpha as oral inhalant, interferon-

alpha as injectable compositions, interferon-alpha as a topical composition, Roferon® analogues, Intron® analogues, Alferon® analogues, and Infergen® analogues, Omniferon® analogues, Alfacon-1 analogues, interferon beta, Avonex™, Betaseron™, Betaferon™, Rebif™, interferon-beta analogues, pegylated interferon-beta, polymerized interferon-beta, dimerized interferon-beta, interferon-beta conjugated to carriers, interferon-beta as oral inhalant, interferon-beta as an injectable composition, interferon-beta as a topical composition, Avonex™ analogues, Betaseron™, Betaferon™ analogues, and Rebif™ analogues. Alternatively, agents that induce interferon-alpha or interferon-beta production or mimic the action of interferon-alpha or interferon-beta may also be employed. The singular form, "interferon compound", may mean any one or more compounds from the class of interferon compounds.

"MBP" means myelin basic protein.

"Mimetic peptide" means a peptide that biologically mimics active determinants on hormones, cytokines, enzyme substrates, viruses or other bio-molecules, and may stimulate, antagonize or otherwise modulate the physiological activity of natural ligands.

"ND4 mouse model" means a transgenic mouse model for multiple sclerosis, produced by transformation with multiple copies of DM20; the genetic mouse model for multiple sclerosis.

"PBS" or "phosphate buffer saline" means an injectable solution that serves as a negative control because it does not have any physiological or therapeutic effects.

"PLP" means an isoprotein proteolipid protein. PLP becomes predominant in the adult.

"Proliferative diseases" means a class of diverse diseases and disorders characterized by a lack of control or poorly controlled cell division or proliferation. Proliferative diseases include anal cancer, bile duct cancer, colon cancer, esophageal cancer, gallbladder cancer, pancreatic cancer, small intestine cancer, stomach cancer, osteosarcoma, ovarian epithelial cancer, gestational trophoblastic tumor, uterine sarcoma, vaginal cancer, vulvar cancer, ovarian germ cell tumor, soft tissue sarcoma, acute lymphoblastic leukemia, acute myeloid leukemia, small cell lung cancer, malignant mesothelioma, malignant thymoma, hypopharyngeal cancer, laryngeal cancer, nasopharyngeal cancer, oropharyngeal cancer,

parathyroid cancer, salivary gland cancer, brain tumor, glioma, cerebellar astrocytoma, cerebral astrocytoma, ependymoma, medulloblastoma, adrenocortical carcinoma, pituitary tumor, islet cell carcinoma, bladder cancer, kidney cancer, penile cancer, Wilm's tumor, AIDS-related lymphoma, cutaneous T-cell lymphoma, hodgkin's lymphoma, Ewing's

5 sarcoma, chronic myelogenous leukemia, hemangiomas of infancy and childhood, mycosis fungoides, hairy cell leukemia, Kaposi's sarcoma, non-hodgkin's lymphoma, multiple myeloma, basal cell carcinoma, malignant melanoma, colorectal cancer, non-small cell lung carcinoma, bladder cancer, pancreatic carcinoma, renal cell carcinoma, neuroblastoma, bladder cancer, breast cancer, cervical cancer, liver cancer, sarcomas, thyroid cancer,

10 endometrial cancer, uterine cancer, multiple myeloma, testicular cancer, retinoblastoma, colorectal cancer, oral cancer, rectal cancer, prostate cancer, restenosis, arteriosclerosis, proliferative diabetic retinopathy. The singular form "proliferative disease" includes any one or more diseases selected from the class of proliferative diseases, and includes any compound or complex disease state wherein a component of the disease state includes a disease selected

15 from the class of proliferative diseases.

"PTX" or "pertussis toxin" means the major protein toxin produced by virulent strains of *Bordetella pertussis*, the organism that causes whooping cough. PTX is a potent ancillary adjuvant that primes macrophages used to elicit several different autoimmune diseases,

20 including EAE.

"RHAMM" means the receptor for hyaluronic acid mediated motility.

"RHAMM-related peptides" refers to peptide sequences or related peptide sequences of

25 RHAMM, specifically, the S-3 peptide, the S-7 peptide, the P-32 peptide, the P-16 peptide, the V-2 peptide, and the V-3 peptide, as defined herein.

"S-3 peptide" means a specific large peptide fragment of RHAMM, and includes the C-terminal or RHAMM and has the approximate size of 45Kda. The term "S-3 peptide" can

30 refer to either the mouse or human amino acid RHAMM peptide fragment, as follows:

**SEQ ID No. 1 Mouse S-3 (333 amino acids)**

AQAILIAQEKYNDTAQSLRDVTAQLESVQEKYNDTAQSLRDVTAQLESEQEKYNDT  
AQSLRDVTAQLESEQEKYNDTAQSLRDVTAQLESVQEKYNDTAQSLRDVSAQLESY

KSSTLKEIEDLKLENLTQEKVAMAEKSVEDVQQQILTAESTNQEYARMVQDLQNRS  
 TLKEEEIKEITSSFLEKITDLKNQLRQQDEDFRKQLEEKGKRRTAEKENVMTELMEIN  
 KWRLLYEELYEKTTPFQQQLDAFEAEKQALLNEHGATQEQLNKIRDSYAQLLGHQN  
 LKQKIKHVVKLKDENSQKSEVSKLRSQLVKRKQNELRLQGELDKALGIR

5

**SEQ ID No 2 Human S-3 (242 amino acids)**

QEKYDSMVQSLEDVTAQFESYKALTASEIEDLKLENSSLQEKAAGKNAEDVQHQI  
 LATESSNQEYVRMLLDLQTKSALKETEIKEITVSFLQKITDLQNQLKQDEDFRKQLE  
 DEEGRKAEKENTTAELTEEINKWRLLYEELYNKTKPFQIQQLDAFEVEKQ  
 10 ALLNEHGAAQEQLNKIRDSYAKLLGHQNLKQKIKHVVKLKDENSQKSEVSKLRQC  
 LAKKKQSETKLQEELNKVLGIK

"S-7 peptide" means a specific RHAMM peptide fragment with an approximate size of 23  
 Kda and represents a smaller portion of S-3 peptide. The term "S-7 peptide" can refer to  
 15 either the mouse or human amino acid RHAMM peptide fragment, as follows:

**SEQ ID No 3 Mouse S-7 (221 amino acids)**

KSSTLKEIEDLKLENLTQEKVAMAEKSVEDVQQQILTAESTNQEYARMVQDLQNRS  
 TLKEEEIKEITSSFLEKITDLKNQLRQQDEDFRKQLEEKGKRRTAEKENVMTELMEIN  
 20 KWRLLYEELYEKTTPFQQQLDAFEAEKQALLNEHGATQEQLNKIRDSYAQLLGHQN  
 LKQKIKHVVKLKDENSQKSEVSKLRSQLVKRKQNELRLQGELDKALGIR

**SEQ ID No 4 Human S-7 (221 amino acids)**

KALTASEIEDLKLENSSLQEKAAGKNAEDVQHQILATESSNQEYVRMLLDLQTKS  
 25 ALKETEIKEITVSFLQKITDLQNQLKQDEDFRKQLEDEEGRKAEKENTTAELTEEINK  
 WRLLYEELYNKTKPFQIQQLDAFEVEKQALLNEHGAAQEQLNKIRDSYAKLLGHQNL  
 KQKIKHVVKLKDENSQKSEVSKLRCQLAKKKQSETKLQEELNKVLGIK

"P-32 peptide" means a specific RHAMM peptide fragment. The term "P-32 peptide" can  
 30 refer to either the mouse or human amino acid RHAMM peptide fragment, as follows:

**SEQ ID No 5: Human P-32**

KQKIKHVVKLKDENSQKSEVSKLRCQLAKKK

**SEQ ID No 6 Mouse P-32**

KQKIKHVVKLKDENSEQLKSEVSKLRSQVVKRK

- 5 "V-2 peptide" means a specific RHAMM peptide fragment which represents a large portion of the original RHAMM peptide, includes the C-terminal and has the approximate size of 58 Kda. The term "V-2 peptide" can refer to either the mouse or human amino acid RHAMM peptide fragment, as follows:

**SEQ ID No 7 Mouse V-2**

- 10 MQILTERLALERQEYEKLQQKELQSQSLLQQEKELSARLQQQLCSFQEEMTSEKNVF  
KEELKLALAE LDAVQQKEEQSERLVKQLEEEERKSTAEQLTRLDNLLREKEVELEKHI  
AAHAQAILIAQEKYNDTAQSLRDVTAQLESVQEKYNDTAQSLRDVTAQLESEQEKY  
NDTAQSLRDVTAQLESEQEKYNDTAQSLRDVTAQLESVQEKYNDTAQSLRDVSAQL  
ESYKSSTLKEIEDLKLENLTQKEKVAMAEKSVEDVQQQILTAESTNQEYARMVQDLQ  
15 NRSTLKEEEEIKEITSSFLEKITDLKNQLRQQDEDFRKQLEEKGKRRTAEKENVMTELT  
EINKWRLLYEELYEYKTKPFQQQLDAFEAEKQALLNEHGATQEQLNKIRDSYAQLLGH  
QNLKQKIKHVVKLKDENSEQLKSEVSKLRSQVVKRKQNELRLQGELDKALGIRHFDPS  
KAFCHASKENFTPLKEGNPNCC

**20 SEQ ID NO 8 Human V-2**

- MQNLKQKFILEQQEHEKLQQKELQIDSLQQEKELSSSLHQKLCSFQEEMVKEKNLF  
EEELKQTLDELDKLQQKEEQAERLVKQLEEEAKSRAEELKLLEEKLGKEAELEKSS  
AAHTQATLLLQEKYDSMVQSLEDVTAQFESYKALTASEIEDLKLENSSLQEKAAG  
KNAEDVQHQILATESSNQEYVRMLLDLQTKSALKETEIKEITVSFLQKITDLQNQLKQ  
25 QEEDFRKQLEDEEGRKA EKENTTAELTEEINKWRLLYEELYNKTKPFQLQLDAFEVE  
KQALLNEHGAAQEQLNKIRDSYAKLLGHQNLKQKIKHVVKLKDENSEQLKSEVSKLR  
CQLAKKKQSETKLQEELNKVLGKHFDPKAFHHESKENFALKTPLKEGNTNCRAP  
MECQESWK

- 30 "V-3 peptide" means a specific RHAMM peptide fragment. The term "V-3 peptide" can refer to either the mouse or human amino acid RHAMM peptide fragment, as follows:

**SEQ ID No 9 Human V-3**

MQNLKQKFILEQQEREKLQQKELQIDSLQQEKELSSSLHQKLCSEFQEEMAKEKNLFE  
 EELKQTLDELDKLQQKEEQAERLVKQLEEEAKSRAEELKLEBKLGKEAELEKSSA  
 AHTQATLLQEKYDSMVQSLEDVTAQFESYKALTASEIEDLKLENSSLQEKAVAKAG  
 KNAEDVQHQLATESSNQEYVRMLLDLQTKSALKETEIKEITVSFLQKITDLQNQLKQ  
 5 QEEDFRKQLEDEEGRKA EKENTTAELTEEINKWRLLYEELYNKTKPFQLQLDAFEVE  
 KQALLNEHGAAQEQLNKIRDSYAKLLGHQNLKQKIKHVVKLKDENSQKSEVSKLR  
 CQLAKKTK

**SEQ ID No 10 Mouse V-3**

10 MQILTERLALERQEYEKLQQKELQSQSLLQQEKELSARLQQQLCSFQEEMTSEKNVF  
 KEELKLALAE L DAVQQKEEQSERLVKQLEBERKSTAEQLTRLDNLLREKEVELEKHI  
 AAHAQAILIAQEKYNDTAQSLRDVTAQLESVQEKYNDTAQSLRDVTAQLESEQEY  
 NDTAQSLRDVTAQLESEQEYNDTAQSLRDVTAQLESVQEKYNDTAQSLRDVSAQL  
 ESYKSSTLKEIEDLKLENLTLQEKVAMAEKSVEDVQQQILTAESTNQEYARMVQDLQ  
 15 NRSTLKEEEIKEITSSFLEKITDLKNQLRQQDEDFRKQLEEKGKRRTAEKENVMTELT  
 EINKWRLLYEELYEKT KPFQQQLDAFEAEKQALLNEHGATQEQLNKIRDSYAQLLGH  
 QNLKQKIKHVVKLKDENSQKSEVSKLRSQLVKRKQN

"P-16 peptide", "P-16", and "Ha binding peptide" mean a 16 amino acid synthetic peptide  
 20 which can bind hyaluronic acid with high affinity. The peptide was isolated using phage  
 display technology. The peptide has the following amino acid sequence:

**SEQ ID No 11 P-16**

CSTMMSRSHKTRSHHV

25

"SJL/J" means a mouse model. Specifically, a female SJL/J mouse has increased  
 susceptibility to development of autoimmune disease. A SJL/J transgenic mouse strain is  
 susceptible to induction of EAE (more susceptible to development of EAE than most other  
 mouse strains). Tumor development as well as autoimmunity in this mouse may result from  
 30 an effective amplification of the immune response.

"Synergistic" means a greater anti-inflammatory, anti-proliferative and/or anti-viral effect  
 with the use of a combination therapy of vitamin B12 and anti-inflammatory, anti-  
 proliferative, and/or anti-viral compounds than with the use of any of these therapeutic



compounds alone. This synergistic effect can work through either similar or different mechanisms or pathways of action. One advantage of a combination therapy with a synergistic effect is that standard dosages can be used for a greater therapeutic effect than expected from the addition of the effect of either compound administered alone; or  
5 alternatively lower dosages or reduced frequency of administration of the therapeutic compound(s) may be used to achieve a better therapeutic effect.

"Viral diseases" means a class of diverse diseases and disorders caused by or believed to be caused by viruses. The class of viral diseases includes genital warts (HPV), HIV/AIDS,  
10 herpes, influenza, measles, polio, varicella-zoster, hepatitis A, hepatitis B, hepatitis C, hepatitis D, hepatitis E, hepatitis G., meningitis, genital warts (HPV), vesicular stomatitis virus infection, and dengue fever. The singular form "viral disease" includes any one or more diseases selected from the class of viral diseases, and includes any compound or complex disease state wherein a component of the disease state includes a disease selected from the  
15 class of viral diseases.

"Vitamin B12 compounds" means a class of compounds which includes vitamin B12 and its analogues, derivatives or conjugates. The class of vitamin B12 compounds includes cyanocobalamin (CN-Cbl), aquacobalamin, adenosylcobalamin, methylcobalamin,  
20 hydroxycobalamin (HC), cyanocobalamin carbanalide, and 5-o-methylbenzylcobalamin [(5-OmeB-za)CN-Cbl] as well as the desdimethyl, monoethylamide and the methylamide analogues of all of the above. Also included are the various analogues and homologues of cobamamide such as coenzyme B12 and 5-deoxyadenosylcobalamin. Other analogues include chlorocobalamin, sulfitocobalamin, nitrocobalamin, thiocyanatocobalamin, benzimidazole  
25 derivatives such as 5,6-dichlorobenzimidazole, 5-hydroxybenzimidazole, trimethylbenzimidazole, as well as adenosylcyanocobalamin [(Ade) CN-Cbl], cobalamin lactone, cobalamin lactam and the anilide, ethylamide, monocarboxylic and dicarboxylic acid derivatives of vitamin B12 or its analogues. Preferred derivatives of vitamin B12 include the mono-, di- and tricarboxylic acid derivatives or the propionamide derivatives of vitamin B12.  
30 In addition, the compositions include polymers of these analogues or vitamin B12 conjugated to other molecules or encapsulated. The singular form, "vitamin B12 compound", may mean any one or more compounds from the class of vitamin B12 compounds.

Figure 1 is a graph showing the effect of vitamin B12, interferon-beta and the combination of vitamin B12 and interferon-beta on clinical scores in the ND4 mouse model.

5

Figure 2 is a graph showing the effect of vitamin B12, interferon-beta and the combination of vitamin B12 and interferon-beta on clinical scores in the EAE mouse model.

Figure 3 is a graph showing the effect of vitamin B12, P-16 and the combination of vitamin B12 and P-16 on clinical scores in the ND4 mouse model.

10

Figure 4 is a graph showing the GFAP staining in brain sections from normal, ND4-untreated animals, interferon-beta treated ND4 animals, and interferon-beta/vitamin B12 treated ND4 animals.

15

Figure 5 is a graph showing the effect of vitamin B12, Taxol™ and the combination of vitamin B12 and Taxol™ on clinical scores in the ND4 mouse model.

20

**Detailed Description of the Invention**

The current invention discloses the effect of vitamin B12 compounds alone and in combination with anti-viral, anti-inflammatory or anti-proliferative compounds for treatment of viral, inflammatory or proliferative diseases. Vitamin B12 compounds alone show efficacy and clearly show an enhancing or synergistic effect in combination with other anti-viral, anti-inflammatory, and anti-proliferative compounds for treating viral, inflammatory, and proliferative diseases.

**10    *Pharmaceutical composition***

The present invention provides pharmaceutical compositions for enhancing anti-viral, anti-proliferative and anti-inflammatory effects and pharmaceutical compositions for treatment of viral, proliferative and inflammatory diseases. The pharmaceutical composition comprises a first compound that is at least one of a vitamin B12 compound and a second compound that is an anti-viral, anti-proliferative and/or anti-inflammatory compound.

***Excipients***

The pharmaceutical compositions of the invention preferably contain a pharmaceutically acceptable carrier or excipient suitable for rendering the compound or mixture administrable orally, intranasally, or parenterally, intravenously, intradermally, intramuscularly or subcutaneously, rectally, via inhalation or via buccal administration, or transdermally. The active ingredients may be admixed or compounded with any conventional, pharmaceutically acceptable carrier or excipient. It will be understood by those skilled in the art that any mode of administration, vehicle or carrier conventionally employed and which is inert with respect to the active agents may be utilized for preparing and administering the pharmaceutical compositions of the present invention. Illustrative of such methods, vehicles and carriers are those described, for example, in *Remington's Pharmaceutical Sciences*, 4th ed. (1970). Those skilled in the art, having been exposed to the principles of the invention, will experience no difficulty in determining suitable and appropriate vehicles, excipients and carriers or in compounding the active ingredients therewith to form the pharmaceutical compositions of the invention.

The compositions of the invention may also be conjugated to transport molecules, monoclonal antibodies or transport modalities such as vesicles and micelles that preferentially target recipient cells.

5    ***Administration***

The compounds of the present invention in the described dosages are administered orally, intranasally, by inhalation, intraperitoneally, subcutaneously, intra-muscularly, transdermally, sublingually or intravenously. For oral administration the pharmaceutical composition can be prepared, for example, in the form of tablets, troches, capsules, elixirs, suspensions, syrups,  
10    wafers, chewing gum or the like prepared by procedures known to those skilled in the art. The amount of active compound in such therapeutically useful compositions or preparations is such that a suitable dosage will be obtained.

The therapeutically effective amount of active agent to be included in the pharmaceutical composition of the invention depends, in each case, upon several factors, e.g., the type, size  
15    and condition of the patient to be treated, the intended mode of administration, the capacity of the patient to incorporate the intended dosage form, the severity of the disease progression, etc.

The dosages used for each anti-viral, anti-proliferative and anti-inflammatory compound are similar to those dosages known to those skilled in the art and used in pre-clinical and clinical studies and in commercial use. The concentrations may be lower than the currently used dosages as the combination of these compounds with vitamin B12 compounds increases efficacy of these compounds. Indeed, vitamin B12 compounds may be combined with anti-  
25    viral, anti-proliferative and anti-inflammatory compounds with the objective to reduce the dosages and/or reduce frequency of administration of the anti-viral, anti-proliferative and anti-inflammatory compounds, in order to achieve both effective treatment and to lessen the negative effects of the anti-viral, anti-proliferative and anti-inflammatory compounds.

The preferred dosage of a vitamin B12 compound for the present invention is the maximum a patient requires to provide an optimal enhancing effect, such maximum being tempered by the absolute upper limit of vitamin B12 dosage being the maximum that a patient can tolerate and not develop any serious complications. Vitamin B12 has been available for many years as an injectable treatment for pernicious anemia, with doses typically in the range of 1000µcg.  
30

Vitamin B12 also has a long history as a general oral health supplement with doses also in the "µg" range. Hydroxocobalamin, a form of vitamin B12, is available as a cyanide poisoning antidote, called Cyanokit® (US Patent # 5,834,448). Cyanokit® is an acute one-time 5-gram dose of hydroxocobalamin administered for emergency purposes. Though vitamin B12 has  
5 been proposed for use for the therapeutic treatment for a few inflammatory diseases, no person has demonstrated that high doses of B12 in combination with an anti-viral, anti-inflammatory or anti-proliferative compound would achieve an enhanced therapeutic effect. With our experimental studies, no toxicity has been found in animals, even at concentrations of 15 mg/kg of vitamin B12 in mice. In a previous acute toxicity study, no toxicities were  
10 observed in mice and rabbits at doses of vitamin B12 (hydroxocobalamin) up to 1g/kg and 100mg/kg body weight, respectively (Mizoule, 1966). The dosage of vitamin B12 compound for our invention are within the range of 10 mg to 2.5 g daily.

Those skilled in the art will be aware that the amounts of the various components of the  
15 compositions of the invention to be administered in accordance with the invention to a patient will depend upon those factors noted above.

#### ***Methods and uses***

The present invention provides methods to enhance or potentiate anti-viral, anti-proliferative  
20 and anti-inflammatory compound-induced anti-viral, anti-proliferative and anti-inflammatory effects and methods of treating viral, proliferative or inflammatory diseases in patients by administering an amount of vitamin B12 compound in combination with anti-viral, anti-proliferative and/or anti-inflammatory compounds. Vitamin B12 compounds can be administered simultaneously, separately or in combination with interferon compounds, under  
25 different dose and route regimens, to enhance the efficacy of anti-viral, anti-proliferative and anti-inflammatory compounds in the treatment of viral, proliferative or inflammatory diseases in patients compared to when such compounds are administered alone.

The first method of treatment is the administration of a pharmaceutical composition including  
30 both a vitamin B12 compound and an anti-viral, anti-proliferative and anti-inflammatory compound. An alternate method of treatment includes the step of the administration of a pharmaceutical composition including a vitamin B12 compound followed by the step of the administration of a second pharmaceutical composition including at least one of an anti-viral, anti-proliferative and anti-inflammatory compound. Optionally, the administration of the

vitamin B12 compound can follow the administration of at least one of the anti-viral, anti-proliferative and anti-inflammatory compound. Optionally, the administration of the pharmaceutical compositions can occur separately or simultaneously

5 The following are examples of acceptable regimens:

1. More than once daily, daily, more than once weekly, weekly, more than  
once monthly or monthly mixtures of vitamin B12 compound in  
combination with anti-viral, anti-proliferative and anti-inflammatory  
compounds for the effective treatment of anti-viral, anti-proliferative and  
anti-inflammatory diseases;  
10
2. More than once daily, daily, more than once weekly, weekly, more than  
once monthly or monthly mixtures of vitamin B12 compound  
simultaneously with anti-viral, anti-proliferative and anti-inflammatory  
compounds for the effective treatment of anti-viral, anti-proliferative and  
anti-inflammatory diseases;  
15
3. More than once daily, daily, more than once weekly, weekly, more than  
once monthly or monthly treatments with anti-viral, anti-proliferative and  
anti-inflammatory compounds and vitamin B12 compounds administered  
separately either more than once daily, daily, more than once weekly,  
weekly, more than once monthly, or monthly;  
20
4. More than once daily, daily, more than once weekly, weekly, more than  
once monthly or monthly mixtures of anti-viral, anti-proliferative and anti-  
inflammatory compounds and vitamin B12 compounds, as well as adjunct  
administration of more than once daily, daily, more than once weekly,  
weekly, more than once monthly or monthly doses of vitamin B12  
compounds.  
25

Those skilled in the art will readily understand that the pathologies and disease states  
expressly stated herein are not intended to be limiting. Rather the compounds of the present  
invention may be used to treat any disease that elicits an inflammatory, proliferative or viral  
response.  
30

**EXAMPLES**

The examples below are exemplary of the embodiments of the present invention.

5     **(A) Examples demonstrating anti-inflammatory effects of interferon-based combination therapies with vitamin B12 compounds**

*Example 1: Effect of vitamin B12, interferon-beta and combination of vitamin B12 incorporating interferon-beta in ND4 mouse model*

***Rationale:***

10     Vitamin B12 and interferon-beta, an anti-inflammatory compound that is used for the treatment of inflammatory diseases such as MS, were used in a demyelinating transgenic mouse model to demonstrate a synergistic effect when a combination of vitamin B12 and anti-inflammatory compounds were used in the treatment of such diseases as compared to treatment with anti-inflammatory compounds or vitamin B12 alone.

15

***Experimental Protocol:***

The ND4 model was used, as a slow progressive demyelinating transgenic mouse model where the animals demonstrate symptoms in young adults at approximately 3 months of age. In this model, the severity of the clinical signs increased until a maximum around 6 months with animals dying around 8 to 9 months of age. Clinical signs assessed included general shaking, seizures, head jerk, hind limb and tail shiver, wobbly gait and limp tail. The scale of zero (absence) to four (constant and uncontrollable movements) was used for each of the clinical signs.

20     Mice (4 animals per group) were treated with vitamin B12 (cyanocobalamin) alone at a dose of 15 mg/kg; weekly and in combination with interferon-beta 1a (Rebif™). Interferon-beta 1a was administered alone and in combination with vitamin B12 at a dose of interferon-beta 1a of 5000 IU, tri-weekly. All treatments began when the mice reached 3 months of age at which time signs of demyelinating disease were evident. Treatment was stopped at time of sacrifice.

25     ***Results and Conclusions:***

Treatment of the ND4 mice with interferon-beta or vitamin B12 showed attenuation of clinical signs of MS, an inflammatory disease (Figure 1) but the combination therapy of interferon-beta and vitamin B12 had a synergistic effect in reducing the clinical signs of the inflammatory disease as compared to treatment with interferon-beta or vitamin B12 alone.

***Example 2: Effect of vitamin B12, interferon-beta and combination of vitamin B12 with interferon-beta in EAE mouse model***

***Rationale:***

- 5 Vitamin B12 and interferon-beta, an example of an anti-inflammatory compound that is used for the treatment of inflammatory diseases such as multiple sclerosis, were used in an autoimmune EAE model to demonstrate an enhancing effect when a combination of vitamin B12 and anti-inflammatory compounds were used in the treatment of such diseases as compared to treatment with either anti-inflammatory compounds or vitamin B12 alone.

10

***Experimental Protocol:***

- Experimental Allergic Encephalomyelitis (EAE) was induced in 7-8 week old female SJL mice. Each mouse was injected intravenously with 200 ng Pertussis Toxin (PT) in 100µl total volume at a tail vein on day 1. Following 48 hours, 200 ng PT was injected a second time.
- 15 Tail vein injections were done with 30.5 gauge needles. Following the PT injection was a one time MBP/CFA (Becton Dickinson) emulsion injection. Each mouse was injected subcutaneously using a 27.5 gauge needle with 200µg/50µl of MBP emulsified in 50ul of complete Freund's adjuvant (CFA) at the base of the tail. The total injection volume was 100µl. The mice were observed daily after first injection and the clinical signs of disease
- 20 were recorded. These included unsteady gait, shaking, tail drop, weight loss, etc. Clinical scores were evaluated every other day starting on day 7 following immunization. Animals were treated daily for 11 days.

- Mice (4 animals per group) were treated with vitamin B12 (cyanocobalamin) alone at a dose
- 25 of 10 mg/kg; daily and in combination with interferon-beta. Interferon-beta 1a (Rebif™) was administered alone and in combination with vitamin B12 at a dose of 5000 IU of interferon-beta, daily; Vitamin B12 and interferon-beta were administered as separate injections starting on the day of first immunization. Treatment was stopped at time of sacrifice. Mice were monitored daily from day 7 after immunization for clinical signs of EAE and were scored on a
- 30 scale of 0 to 5. A score of 0 represented the absence of signs while a score of 5 was given to moribund animals.

***Results and Conclusions:***



Treatment of the EAE mice with interferon-beta alone showed attenuation of clinical signs of the inflammatory disease (Figure 2) but the combination therapy of interferon-beta and vitamin B12 were more effective in reducing the clinical signs of the inflammatory disease, indicating the combination of interferon-beta and vitamin B12 enhanced the anti-inflammatory activity of interferon-beta.

***Example 3: Effect of interferon concentration on B12 synergistic effects***

***Rationale:***

Vitamin B12 (cyanocobalamin) and varying concentrations of interferon-beta, an example of anti-inflammatory compound used for the treatment of inflammatory diseases such as multiple sclerosis, were used in the ND4 transgenic model described above to demonstrate concentration effect of anti-inflammatory compounds on B12 synergistic effects.

***Experimental Protocol:***

ND4 mice were either (a) untreated or treated with (b) interferon-beta 1a (Rebif™) alone (5000 IU) or (c) 1000 IU of interferon-beta 1a and B12 (15mg/kg), or (d) 2500 units of interferon-beta 1a and B12 (15mg/kg) or (e) 5000 IU of interferon-beta 1a and B12 (15mg/kg), three times a week for the duration of the experiment. Treatment was started when the animals were 3 months of age and continued for 11 weeks. The animals were evaluated twice a week for clinical signs and the scores were averaged on a weekly basis. Refer to example 1 for more experimental details.

***Results and Conclusions:***

The results demonstrated that the effectiveness of the combination therapy was dependent on the concentration of interferon-beta, with significant reduction of clinical scores (at interferon-beta concentration of 5000 IU). Thus, by combining vitamin B12 and interferon-beta, a lower level of interferon was needed to achieve pharmacological action. (Table 1).

**Table 1. Effect of interferon-beta concentration on vitamin B12 synergistic effect on the clinical signs and symptoms in the transgenic mouse at six months**

Treatment	Sum of Clinical Scores (+/- SD)
ND4 Untreated	22 +/- 2
interferon-beta (5000 IU)	21 +/- 4
interferon-beta (1000 IU) + Vitamin B12 (15mg/kg)	18 +/- 1

interferon-beta (2500 IU) + Vitamin B12 (15mg/kg)	16 +/- 1
interferon-beta (5000 IU) + Vitamin B12 (15mg/kg)	13 +/- 2

***Example 4: Effect of vitamin B12, RHAMM mimetic (P-16 peptide) and combination of vitamin B12 with P-16 peptide in ND4 mouse model***

***Rationale:***

- 5 P-16 peptide, a HA mimetic peptide, had been demonstrated to possess anti-inflammatory properties in both MS and diabetes animal studies. The effects of vitamin B12 in combination with P-16 peptide (an example of an anti-inflammatory compound) in the treatment of inflammatory diseases was investigated.

10 ***Experimental Protocol:***

- The ND4 model is a slow progressive demyelinating transgenic mouse model where the animals demonstrate symptoms in young adults at approximately 3 months of age. The severity of the clinical signs increase until a maximum around 6 months with animals dying around 8 to 9 months of age. Clinical signs assessed include general shaking, seizures, head  
15 jerk, hind limb and tail shiver, wobbly gait and limp tail. The scale of zero (absence) to four (constant and uncontrollable movements) was used for each of the clinical signs.

- Mice (4 animals per group) were treated with vitamin B12 (cyanocobalamin) alone at a dose of 15 mg/kg; weekly and in combination with P-16. P-16 was administered alone and in  
20 combination with vitamin B12 at a dose of 1 mg/kg, tri-weekly. All treatments began when the mice reached 3 months of age at which time signs of demyelinating disease were evident. Treatment was stopped at time of sacrifice.

***Results and Conclusions:***

- 25 Treatment of the ND4 mice with the P-16 peptide showed attenuation of clinical signs of MS, an inflammatory disease (Figure 3) but the combination therapy of P-16 and vitamin B12 was more effective in reducing the clinical signs of the inflammatory disease than P-16 alone.

**(B) Examples demonstrating anti-proliferative effects of combination therapies incorporating vitamin B12 compounds**

**Example 5: Effect of vitamin B12, interferon and combination of vitamin B12 with interferon in inhibiting cellular proliferation in vitro**

**Rationale:**

Interferon was also known as an anti-proliferative agent that has been reported to be effective in treating proliferative diseases such as Kaposi's sarcoma, melanoma, fibrosarcoma and high-grade astrocytoma (Fine et al., 1997). The following experiment was carried out to determine whether vitamin B12 could enhance the anti-proliferative effects of an anti-inflammatory compound such as interferon in astrocytoma cell lines.

**Experimental Protocol:**

Mouse ascite astrocytoma cells were seeded in a 96-well plate and grown to 60% confluence in standard growth conditions. Prior to treatment, cells were washed and the culture medium was replaced with culture medium containing interferon-beta alone (500 IU/ml), vitamin B12 (methylcobalamin)(25 µg/ml) alone, or combination of interferon-beta (500 IU/ml), and vitamin B12 (25 µg/ml), and incubated overnight.

The CellTiter 96<sup>®</sup> AQueous One Solution Cell Proliferation Assay was used as a colorimetric method for determining the number of viable cells in proliferations. Cell proliferation assays were performed by adding a small amount of the CellTiter 96<sup>®</sup> AQueous One Solution Reagent directly to culture wells, incubating for 1-4 hours and then recording absorbance at 490nm with a 96 well plate reader. The quantity of formazan product as measured by the amount of 490nm absorbance is known in the art to be directly proportional to the number of living cells in the culture.

**Results and Conclusions:**

Treatment of either mouse interferon-beta or vitamin B12 (methylcobalamin) alone resulted in a decrease in proliferative activity as compared to untreated cells. However, anti-proliferative activity was significantly increased when cells were treated with a combination of vitamin B12 and interferon-beta (Table 2 below) as compared to treatment with either vitamin B12 or interferon-beta alone.

**Table 2. Effect of combination therapy on cellular proliferation in a mouse astrocytoma cell line**

Treatment	Measure of Proliferative Activity (Absorbance at 490 nm)
No treatment	0.8955
Treatment with Mouse Interferon-beta (500 IU/ml)	0.3855
Treatment with Vitamin B12 (25µg/ml)	0.2985
Treatment with Mouse Interferon-beta (500 IU/ml) and Vitamin B12 (25µg/ml)	0.1435

5

***Example 6: Effect of vitamin B12, interferon, combination of vitamin B12 with interferon on astrocyte gliosis***

**10 *Rationale:***

Astrocyte gliosis refers to the increased reactivity and proliferation of astrocytes in the central nervous system and is thought to play a major role in the development of lesions in the brain. Specifically, astrocyte gliosis has been observed around hematogenous metastases of the human brain and GFAP, the protein marker for reactive astrocytes, has been used as a  
 15 biomedical marker for early detection for chemically induced cancer. Both the ND4 and EAE mouse models were used below to illustrate the effect of the combination therapy on astrocyte gliosis. The ND4 transgenic mice develops normally up to three months, after which demyelination progresses thereafter. Along with primary demyelination, astrogliosis increases from about twice normal levels at three months to extensive astrogliosis by adult stage.  
 20 Similarly, the EAE-induced mice had also been shown to demonstrate extensive astrogliosis.

***(a) ND4 Model***

***Experimental Protocol:***

ND4 mice were treated as described previously in Example 1. After the various treatments,  
 25 the mice were sacrificed and brain sections removed for GFAP staining and quantitation. The brain region of hippocampus, adjacent to the dentate gyrus was fixed in formalin, paraffin-

embedded and sectioned at 5  $\mu$ m. Immunohistochemistry with anti-GFAP antibody was used to stain for GFAP. Glial fibrillary acidic protein was a protein expressed by astrocytes (particularly reactive astrocytes) and was used as a marker for these cells.

- 5 In addition, quantification of GFAP within the brains of normal, ND4 untreated and ND4 treated mice was carried out to provide a more precise level of protein expression. Whole brain homogenates were assayed for GFAP by slot blot. Brains were homogenized in a buffer containing 50 mM Tris-HCl pH 7.6, 0.5 mM DTT, 1 mM EDTA, and 0.43 mM PMSF. The homogenate was centrifuged at 11000 rpm for 30 min at 4°C. The pellet was isolated and  
10 resuspended in a buffer containing 10 mM sodium phosphate pH 7.5, 2 mM DTT, 6 M urea and 1 mM EDTA. Samples were loaded onto the slot blot apparatus (BioDot, Biorad) under vacuum. The blots were reacted with anti-GFAP antibodies and then a secondary antibody. The blots were developed and the relative amounts of each band quantified.

#### 15 ***Results and Conclusions:***

- There was a significant increase in GFAP staining of untreated ND4 animals compared to normal animals (**Figure 4**), indicating extensive astrocyte gliosis. This high level of GFAP staining was decreased in interferon-beta treated animals. Treatment with interferon decreased the number of activated fibrous astrocytes slightly and the levels of GFAP in brain  
20 homogenates. The treatment of interferon-beta and vitamin B12 inhibited the levels of activated astrocytes and levels of GFAP to near normal levels (**Table 3**). These data clearly demonstrated that the combination treatment of interferon-beta (an example of an anti-proliferative compound) with vitamin B12 acted in a synergistic manner to inhibit astrocyte proliferation and activation.

25

**Table 3. Total relative GFAP in brain homogenates from normal, ND4 untreated animals, vitamin B12-treated, interferon-beta treated; and interferon-beta and vitamin B12 treated ND4 animals.**

Treatment	Relative Amount of GFAP (+/- SD)
Normal	1.0
Untreated ND4 mice	4.4 +/- 0.2
Vitamin B12 (15mg/kg)	2.8 +/- 0.1
interferon-beta (5000 IU/ml)	3.1 +/- 0.1
interferon-beta (5000 IU/ml) +Vitamin	1.6 +/- 0.2

B12 (15mg/kg)

**(b) EAE Model****Experimental Protocol:**

5 EAE mice were treated as described previously in Example 2. After the various treatments, the mice were sacrificed and brain sections removed for GFAP staining and quantitation. The brain region of hippocampus, adjacent to the dentate gyrus was fixed in formalin, paraffin-embedded and sectioned at 5  $\mu$ m. Immunohistochemistry with anti-GFAP antibody was used to stain for GFAP. Glial fibrillary acidic protein was a protein expressed by astrocytes  
10 (particularly reactive astrocytes) and was used as a marker for these cells.

In addition, quantification of GFAP within the brains of normal, EAE untreated and EAE treated mice was carried out to provide a more precise level of protein expression. Whole brain homogenates were assayed for GFAP by slot blot. Brains were homogenized in a buffer  
15 containing 50 mM Tris-HCl pH 7.6, 0.5 mM DTT, 1 mM EDTA, and 0.43 mM PMSF. The homogenate was centrifuged at 11000 rpm for 30 min at 4°C. The pellet was isolated and resuspended in a buffer containing 10 mM sodium phosphate pH 7.5, 2 mM DTT, 6 M urea and 1 mM EDTA. Samples were loaded onto the slot blot apparatus (BioDot, Biorad) under vacuum. The blots were reacted with anti-GFAP antibodies and then a secondary antibody.  
20 The blots were developed and the relative amounts of each band quantified.

**Results and Conclusions:**

Treatment with vitamin B12 was not effective in decreasing the levels of GFAP while treatment of interferon-beta was able to reduce the amounts of GFAP by ~50%. However,  
25 treatment of interferon-beta and vitamin B12 was the most effective among the treatment groups. The combination therapy inhibited the levels of activated astrocytes and levels of GFAP to near normal levels (Table 4).

These data clearly demonstrated that the combination treatment of interferon-beta, an example  
30 of anti-proliferative compound, with vitamin B12 acted in a synergistic manner to inhibit astrocyte proliferation and activation.

**Table 4. Total relative GFAP in brain homogenates from normal, EAE untreated animals, vitamin B12 treated interferon-beta treated; and interferon-beta and vitamin B12 treated EAE animals.**

Treatment	Relative Amount of GFAP (+/- SD)
Normal	1.0
Untreated EAE mice	4.2 +/- 0.2
Vitamin B12 (15mg/kg)	4.2 +/- 0.2
interferon-beta (5000 IU/ml)	2.0 +/- 0.5
interferon-beta (5000 IU/ml) +Vitamin B12 (15mg/kg)	1.4 +/- 0.2

5

10

***Example 7: Effect of vitamin B12, Taxol™ and combination of vitamin B12 with paclitaxel (Taxol™) in ND4 mouse model***

***Rationale:***

15 Paclitaxel (Taxol™) was an anti-proliferative agent that is used to treat cancers such as ovarian cancer and breast cancer. The following experiment was carried out to determine whether vitamin B12 could enhance the anti-proliferative effects of anti-proliferative compounds such as Taxol™ in the ND4 mouse model. As discussed earlier, the ND4 mouse model was associated with astrocyte proliferation in the brain.

20 ***Experimental Protocol:***

The ND4 transgenic mice received one of the following treatments through intraperitoneal injections: 1) Taxol™ at a dose of 20 mg/kg once a week; 2) vitamin B12 at a dose of 10 mg/kg once a week and Taxol™ at a dose of 5 mg/kg three times per week; and 3) vitamin B12 at a dose of 10 mg/kg once a week. All treatments started when the mice reached 3  
25 months of age, at which time signs of demyelinating disease were evident.

***Results and Conclusions:***

Treatment of the ND4 mice with the vitamin B12 or Taxol™ alone showed a significant attenuation of mean clinical scores. The combination therapy of vitamin B12 with Taxol™ was more effective in attenuation of clinical signs compared to when these compounds were used alone. There was an enhanced therapeutic effect when vitamin B12 and Taxol™ were

5 combined. Results are shown in Figure 5.



**(C) Examples demonstrating anti-viral effects of combination therapies with vitamin B12**

**Example 8: *In vitro* studies demonstrating the enhancing effect of interferon compound with vitamin B12 compounds on treatment of human hepatitis B virus (HBV)**

***Rationale:***

Hep G2.2.15 was used as a HepG2 cell line that is stably transfected with HBV genome and that continues to secrete HBV virions into the cell growth medium. This cell line has been widely used to study the activities of anti-viral agents against HBV infection and was used in this study to evaluate the enhancing effect of vitamin B12 compounds with interferon, an example of an anti-viral compound, on treating HBV infections. Interferon was used as an example of an anti-viral agent because it was used to treat both hepatitis B and C in the clinic.

***Experimental Protocol:***

HepG2.2.15 cells, a cell line that was stably transfected with HBV genome, were cultured in RPMI-1640 with fetal bovine serum at either 2 or 4% final concentration and with no antibiotics. The cells were seeded in a 96-well plate at a density of  $2 \times 10^4$  cells/well and grown overnight. The medium was then discarded and replaced with 100  $\mu$ l of culture medium containing hydroxocobalamin (a form of vitamin B12) alone (at various concentrations), interferon-beta alone (at various international units), and a mixture of varying doses of hydroxocobalamin (HC) and various doses of interferon-beta vs HC. Triplicate samples were measured for each concentration. The cells were incubated at 37°C for 9 days. Drug/medium was changed daily. On day 9, the culture media containing released HBV virions were harvested and used to extract HBV DNA.

HBV DNA from drug-treated HepG2.2.15 cell culture media was extracted using QIAamp DNA mini kit – blood and body fluid spin column according to manufacturer's instructions. Briefly, 10  $\mu$ l of QIAGEN proteinase K was added to 1.5 ml Eppendorf tube. 50  $\mu$ l cell supernatant and 50  $\mu$ l AL buffer were added to the tube. The samples were incubated at 56°C water bath for 3 h. At the end of incubation, 50  $\mu$ l ethanol was added to the sample. The mixture was transferred to a QIAamp spin column and centrifuged at 14000 rpm for 1 min. The filtrate was discarded. 50  $\mu$ l Buffer AW1 was then added to the column and centrifuged

at 14000 rpm for 1 min. Following washing and recentrifugation, DNA was eluted in 50  $\mu$ l dH<sub>2</sub>O.

HBV DNA was detected by real-time PCR using Lightcycler assay. The reaction was performed in 20  $\mu$ l, comprising the following: 6.4/4.8  $\mu$ l dH<sub>2</sub>O, 2  $\mu$ l 25 mM MgCl<sub>2</sub>, 1  $\mu$ l 10  $\mu$ M sense primer, 1  $\mu$ l 10  $\mu$ M antisense primer, 8/1.6  $\mu$ l LightCycler Probe (fluorescein-labeled, 5 $\mu$ M), 0.8/1.6  $\mu$ l LightCycler Probe 2 (LC Red 640-labeled, 10 $\mu$ M), 2  $\mu$ l LC FastStart DNA Master Hyb Probes, 1  $\mu$ l uracil DNA glycosylase.

PCR mix was aliquoted into glass capillaries and 5  $\mu$ l DNA template was then added to the PCR mix. A range of HBV plasmid DNA ( $1 \times 10^8$ ,  $1 \times 10^7$ ,  $1 \times 10^6$ , ...,  $1 \times 10^2$ , 25 copies) was used as standards for quantitative analysis. The cycling conditions are shown in Table 5.

**Table 5. The cycling conditions for Lightcycler assay**

Program	# cycles	Target Temp	Incubation Time	Temp Transition	Acquisition Mode
Denaturation	1	95°C	9 min	20°C/sec	None
PCR	45	95°C	5 sec	20°C/sec	None
		54°C	10 sec	20°C/sec	Single
		72°C	17 sec	20°C/sec	None
Melt	1	98°C	0 sec	20°C/sec	None
		45°C	15 sec	20°C/sec	None
		70°C	0 sec	0.2°C/sec	Continuous
Cool	1	40°C	30 sec	20°C/sec	None

The antiviral effects of the test compounds were calculated using DNA copy numbers as an endpoint. Specifically, the antiviral activities were calculated based on the following formula: Antiviral effect =  $(C_{\text{control}} - C_{\text{test}})/C_{\text{control}}$  whereby the  $C_{\text{control}}$  is the HBV DNA copies in the control wells (without drug); the  $C_{\text{test}}$  is the HBV DNA copies in the test wells with a given concentration of a test compound.

The enhancing or antagonistic effect was analyzed mathematically using the median effect equation (Chou and Talalay, 1984) by computer software CalcuSyn (Biosoft, St Louis). The range of the combination index (CI) is illustrated below in Table 6.

**Table 6. Correlation between combination index and degree of synergism**

<0.1	Very strong synergism
0.1-0.3	Strong synergism
0.3-0.7	Synergism
0.7-0.85	Moderate synergism
0.9-1.1	Additive
>1.1	Antagonism

### Results and Conclusions:

**Table 7. Antiviral effects of test compounds alone and combinations on HBV**

5 measured by HBV DNA copies using Lightcycler assay

Concentrations ( $\mu\text{g/ml}$ )	0	1	10	100	500
HC	0	0.001	0.001	0.001	0.112
Concentrations (IU/ml)	0	50	100	500	1000
interferon-beta	0	0.569	0.746	0.773	0.823
Concentrations ( $\mu\text{g/ml}$ )/(IU/ml)	0	1/50	10/100	100/500	500/1000
HC / interferon-beta	0	0.571	0.772	0.912	0.851

**Table 8. CI values for the combinations between hydroxocobalamin (HC) and interferon-beta on HBV**

HC ( $\mu\text{g/ml}$ )	interferon- beta (IU/ml)	CI
1	50	1.908
10	100	0.258
100	500	0.051
500	1000	0.572

10 Viral activity was decreased when cells were treated with interferon-beta but not with vitamin B12. The combination therapy of vitamin B12 with interferon-beta was more effective in attenuating viral activity compared to when these compounds were used alone. There was an enhanced effect for the treatment of hepatitis B infection when vitamin B12 and interferon were combined. See Table 7 for more details.

15

Specifically, strong and moderate potentiating effects were examined for HC on interferon-beta activity against HBV at the concentrations were higher than 10  $\mu\text{g/ml}$  of HC and 100 IU/ml of interferon-beta, respectively. Based on the outcome of the combination index

analysis, the anti-viral effects of interferon-beta with vitamin B12 were synergistic. The results are summarized in Table 8 above.

5 **Example 9: *In vitro* studies demonstrating the enhancing effect of interferon anti-viral activity with vitamin B12 on herpes simplex virus type 2 (HSV-2) and vesicular stomatitis virus (VSV)**

***Rationale:***

10 MRC-5 (human fibroblast cells) was a HSV and VSV susceptible cell line that was used widely to study the activities of anti-viral agents against VSV and HSV infections. This cell line was used in this study to evaluate the enhancing effect of vitamin B12 compound with interferon-alpha and interferon beta, both examples of anti-viral compounds, on treating VSV and HSV infections.

15 ***Experimental Protocol:***

A CPE (cytopathic effect) inhibition assay was performed. This assay measures the ability of a drug or agent to protect cells from lysis by a virus. Briefly, confluent MRC-5 cells in a 96-well plate were incubated with various concentrations of hydroxocobalamin (HC) alone, interferon-alpha alone, interferon-beta alone, and the mixtures of varying doses of HC and various doses of interferon-alpha or interferon-beta. The test concentrations for HC were 0, 1, 10, 100, 500 and 1000 µg/ml and that of interferon-alpha and interferon-beta were 0, 5, 10, 50, 100, and 500 IU/ml. Triplicate samples were tested for each concentration in a 96-well plate format. The culture medium for MRC-5 cells was recommended by ATCC (Minimum Essential Medium Eagle with 2 mM L-glutamine and Earle's BSS adjusted to contain 1.5 g/L sodium bicarbonate, 0.1 mM non-essential amino acids, and 1.0 mM sodium pyruvate, 10% fetal bovine serum).

30 After overnight incubation, the cells were infected with either VSV at moi (multiplicity of infection) of 0.5 pfu/cell or HSV-2 at moi of 0.2 pfu/cell at 37°C for 1 h. Following removal of virus inoculum, the infected cells were washed with PBS and covered with medium containing either no or increasing concentrations of test compounds as above for 2-3 days at 37°C until the CPE in the control wells was 100%. The cells protected from CPE were measured using neutral red uptake assay. Specifically, culture medium was removed from cells in a 96-well plate by pump. The cells were then washed once with 200 µl of PBS. 100 µl

of 0.01% neutral red (in PBS) was subsequently added to each well, and incubated at 37°C for 30 min. The dye was then removed and the cells were washed twice with 200µl PBS per well. The dye was extracted by addition of 100 µl of 50% ethanol/1% glacial acetic acid in PBS to each well and incubated at room temperature for 15 min with gentles shaking at 120-150 rpm.

5 The absorbance at 550nm was read on an ELISA reader.

The antiviral effects were calculated based on the following formula: Antiviral effect =  $(OD_{Test} - OD_0)/(OD_{Control} - OD_0)$  whereby  $OD_{Test}$  is the optical density measured with a given concentration of the test compound;  $OD_0$  is the optical density measured at drug concentration zero;  $OD_{Control}$  is the optical density of uninfected cells.

10

The potentiating effect was analyzed mathematically using the median-effect equation (Chou and Talalay, 1984) by a computer program CalcuSyn (Biosoft, St Louis). Refer to Table 6 for correlation between combination index obtained and degree of synergism.

15

### **Results and Conclusions:**

#### **(a) HSV-2 Infection**

The combination therapy of vitamin B12 with interferon-alpha or interferon-beta was more effective in attenuation viral activity compared to when these compounds were used alone (Table 9). There was an enhanced effect for the treatment of herpes simplex virus infection when vitamin B12 and interferon, an anti-viral agent, were combined. Specifically, a strong potentiating effect was observed for HC on interferon-alpha activity against HSV-2 when the concentrations of HC and interferon-alpha were higher than 10 µg/ml and 10 IU/ml respectively. Strong and moderate potentiating effects were observed for HC on interferon-beta activity against HSV-2 at all concentrations tested. Based on the outcome of the combination index analysis, the anti-viral effects of interferon-alpha or interferon-beta with vitamin B12 were synergistic (Table 10).

20

25

30 **Table 9. Antiviral effects of test compounds alone and combinations on HSV-2 determined by CPE inhibition assay**

Concentrations (µg/ml)	0	1	10	100	500	1000
HC	0	0.014	0.032	0.056	0.166	0.210
Concentrations (IU/ml)	0	5	10	50	100	500
interferon-alpha	0	0.093	0.139	0.218	0.282	0.271

interferon-beta	0	0.164	0.136	0.314	0.281	0.265
Concentrations ( $\mu\text{g/ml}$ )/(IU/ml)	0	1/5	10/10	100/50	500/100	1000/500
HC / interferon-alpha	0	0.090	0.257	0.342	0.724	0.999
HC / interferon-beta	0	0.169	0.315	0.368	0.683	0.748

Table 10. CI values for the combinations between HC and interferon-alpha or interferon-beta on HSV-2

HC ( $\mu\text{g/ml}$ )	interferon- alpha (IU/ml)	CI	HC ( $\mu\text{g/ml}$ )	interferon- beta (IU/ml)	CI
1	5	2.292	1	5	0.746
10	10	0.07	10	5	0.026
100	50	0.117	100	50	0.065
500	100	0.009	500	100	0.012
1000	500	<0.01	1000	500	0.013

(b) VSV Infection

- 5 Anti-viral activity (as reflected by protection of CPE) was significantly increased when cells were treated with the vitamin B12 or interferon-alpha or interferon-beta. The combination therapy of vitamin B12 with interferon-alpha or interferon-beta was more effective in attenuation viral activity compared to when these compounds were used alone. See Table 11. There was an enhanced effect for the treatment of vesicular stomatitis virus infection when
- 10 vitamin B12 and interferon, an anti-viral agent, were combined. Specifically, strong potentiating effect was observed for HC on interferon-beta activity against HSV at all concentrations tested and on interferon-alpha activity when the concentrations of HC and interferon-alpha were higher than 100  $\mu\text{g/ml}$  and 50 IU/ml respectively. Based on the outcome of the combination index analysis, the anti-viral effects of interferon-alpha or
- 15 interferon-beta with vitamin B12 were synergistic (Table 12).

Table 11. Antiviral effects of test compounds alone and combinations on VSV determined by CPE inhibition assay

Concentrations ( $\mu\text{g/ml}$ )	0	1	10	100	500	1000
HC	0	0.001	0.001	0.039	0.001	0.002
Concentrations (IU/ml)	0	5	10	50	100	500
interferon-alpha	0	0.296	0.387	0.950	0.996	0.942
interferon-beta	0	0.826	0.985	0.758	0.768	0.956
Concentrations ( $\mu\text{g/ml}$ )/(IU/ml)	0	1/5	10/10	100/50	500/100	1000/500

HC / interferon-alpha	0	0.299	0.450	0.999	0.999	0.999
HC / interferon-beta	0	0.999	0.999	0.999	0.999	0.999

**Table 12. CI values for the combinations between HC and interferon-alpha or interferon-beta on VSV**

HC ( $\mu\text{g/ml}$ )	interferon- alpha (IU/ml)	CI	HC ( $\mu\text{g/ml}$ )	interferon- beta (IU/ml)	CI
1	5	1.6	1	5	0
10	10	1.766	10	5	0
100	50	0.013	100	50	0.002
500	100	0.027	500	100	0.004
1000	500	0.016	1000	500	0.021

## 5 Summary of examples

In summary, these examples clearly demonstrate that the combination treatment of a vitamin B12 compound in conjunction with a therapeutic agent such as interferon, taxol or P-16 is effective in treating inflammatory, proliferative and/or viral diseases and that B12 enhances the effectiveness of the specific therapeutic agent against the disease indicated.

10

Although the invention has been described with preferred embodiments, it is to be understood that modifications may be resorted to as will be apparent to those skilled in the art. Such modifications and variations are to be considered within the purview and scope of the present invention.

15

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**WE CLAIM:**

1. A pharmaceutical composition for the treatment of a disease selected from the group consisting of: viral diseases; proliferative diseases; inflammatory diseases; proliferative and inflammatory diseases; proliferative and viral diseases; viral and inflammatory diseases; and proliferative, viral and inflammatory diseases; comprising:
- (i) At least one vitamin B12 compound; and
- (ii) At least one second compound selected from the group consisting of: an anti-viral compound; an anti-proliferative compound; and an anti-inflammatory compound.
2. A pharmaceutical composition for the treatment of proliferative diseases comprising:
- (i) At least one vitamin B12 compound; and
- (ii) At least one anti-proliferative compound.
3. A pharmaceutical composition for the treatment of viral diseases comprising:
- (i) At least one vitamin B12 compound; and
- (ii) At least one anti-viral compound.
4. A pharmaceutical composition for the treatment of inflammatory diseases comprising:
- (i) At least one vitamin B12 compound; and
- (ii) At least one anti-inflammatory compound.
5. The pharmaceutical composition of Claim 2, wherein the anti-proliferative compound is interferon.
6. The pharmaceutical composition of Claim 3, wherein the anti-viral compound is interferon.

7. The pharmaceutical composition of Claim 4, wherein the anti-inflammatory compound is interferon.
8. A pharmaceutical composition for the treatment of a virus selected from the group consisting of Hepatitis B, hepatitis C, herpes simplex virus type 2 and vesticular stomatitis virus comprising:
- (i) At least one vitamin B12 compound; and
  - (ii) At least one interferon compound.
9. A pharmaceutical composition for the treatment of cancer comprising:
- (i) At least one vitamin B12 compound; and
  - (ii) At least anti-proliferative compound.
10. A pharmaceutical composition for the treatment of astrocytoma and glioma comprising:
- (i) At least one vitamin B12 compound; and
  - (ii) At least anti-proliferative compound.
11. A pharmaceutical composition for the treatment of hepatitis C comprising:
- (i) At least one vitamin B12 compound; and
  - (ii) At least one interferon compound
12. A pharmaceutical composition for the treatment of cancer comprising:
- (i) At least one vitamin B12 compound; and
  - (ii) Paclitaxel.
13. A pharmaceutical composition for the treatment of astrocytoma and glioma comprising:
- (i) At least one vitamin B12 compound; and
  - (ii) Paclitaxel.

14. A pharmaceutical composition for the treatment of astrocytoma and glioma comprising:

- (i) At least one vitamin B12 compound; and
- (ii) At least one interferon compound.

5

15. A pharmaceutical composition for the treatment of multiple sclerosis comprising:

- (i) Vitamin B12; and
- (ii) A peptide selected from the group consisting of P16, S-3, S-7, P-32, V-2 and V-3.

10

16. A pharmaceutical composition for the treatment of Hepatitis B comprising:

- (i) Vitamin B12; and
- (ii) Interferon

15

17. A pharmaceutical composition according to any of claims 1-16 wherein the first compound is conjugated to the second compound.

18. A method of treating a disease selected from the group consisting of viral, proliferative, and inflammatory diseases comprising the step of administering to a patient the pharmaceutical composition of any of claims 1-17.

20

19. A method of treating hepatitis B or hepatitis C comprising the step of administering to a patient the pharmaceutical composition of any of claims 1, 3, 6, 8, 11 and 16.

25

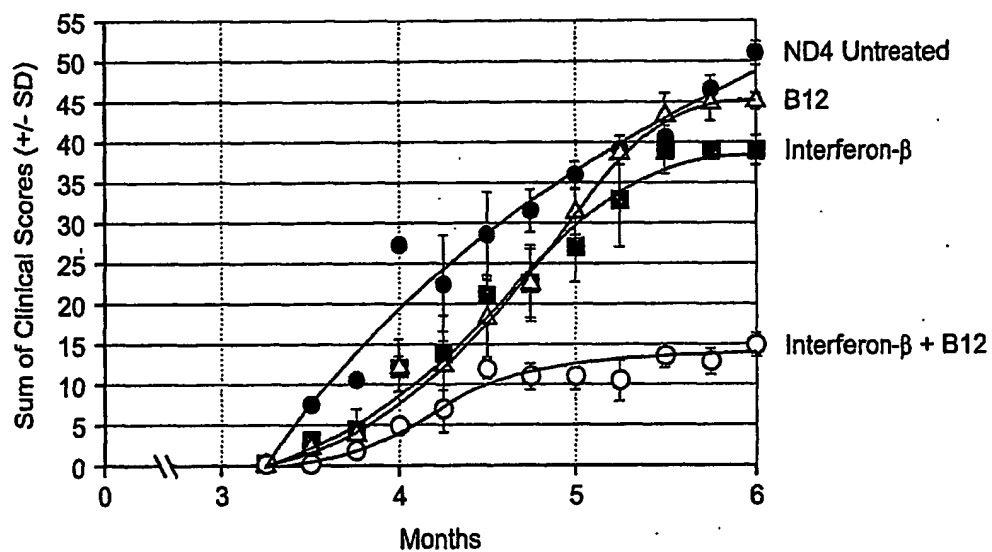
20. A method of treating a disease selected from the group consisting of viral, proliferative and inflammatory diseases comprising the steps of administering to a patient, either together, or separately:

- (a) At least one Vitamin B12 compound; and

(b) At least one compound selected from the group consisting of: an anti-viral compound, an anti-proliferative compound, and an anti-inflammatory compound.

- 5           21. The method disclosed in any of claims 18-20 wherein the dose of Vitamin B12 compound is between 10-2500 mg.
22. The method disclosed in any of claims 18-20 wherein the dose of the anti-viral, anti-proliferative or anti-inflammatory compound is equivalent to the therapeutic dose  
10           range of said anti-viral, anti-proliferative, or anti-inflammatory compound when used alone.
23. The method disclosed in any of claims 18-20 wherein the dose of anti-viral, anti-proliferative or anti-inflammatory compound is less than the therapeutic dose range of  
15           said anti-viral, anti-proliferative, or anti-inflammatory compound when used alone.
24. The use of a compound according to any of claims 1-17 to treat a viral, proliferative, or inflammatory disease.

**Figure 1.** Effect of B12, interferon-beta and the Synergistic Combination of B12 and interferon-beta on Clinical Scores in ND4 Mouse Model



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Figure 2. Effect of B12, interferon-beta and the combination of B12 and interferon-beta on clinical scores in EAE mouse model

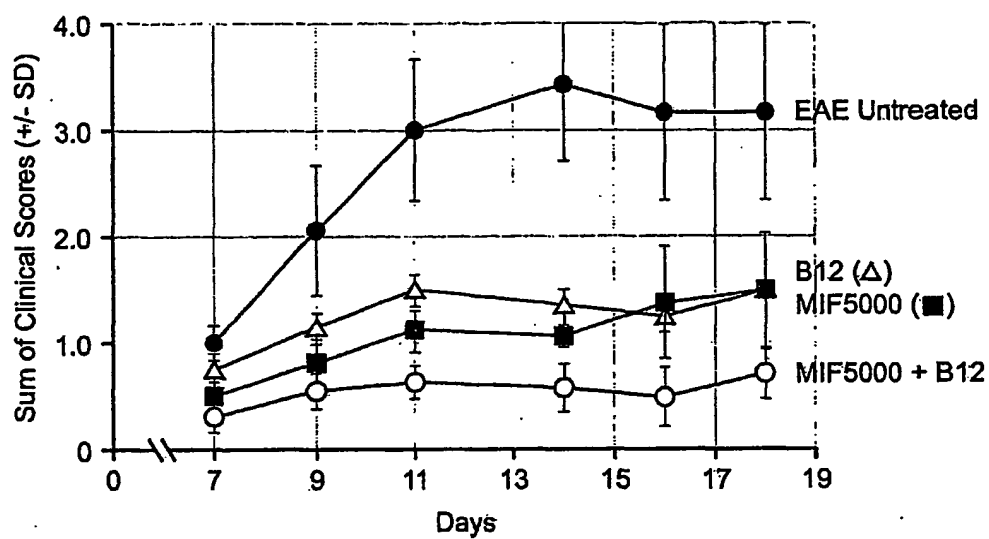
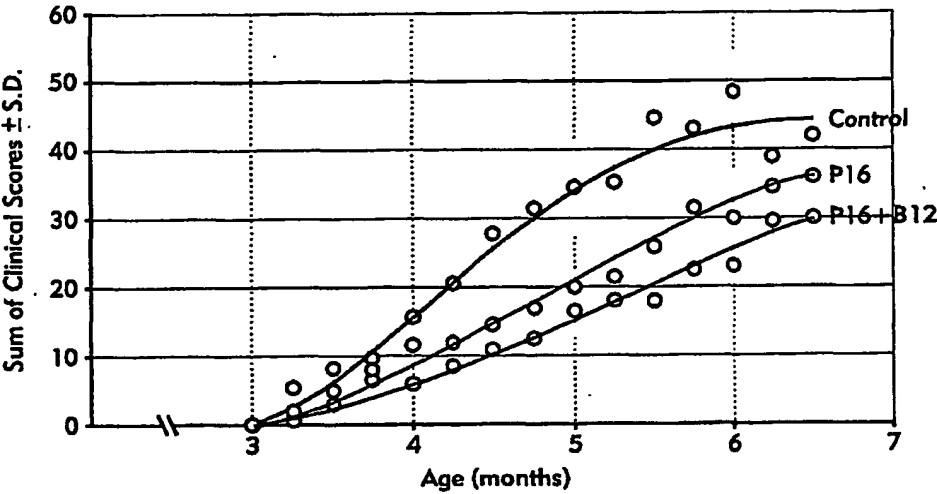




Figure 3. Effect of B12, P-16 and the Combination of B12 and P-16 on Clinical Scores in ND4 Mouse Model



**Figure 4.** GFAP staining in brain sections from normal, ND4 untreated animals, interferon beta treated ND4 animals, and interferon-beta and vitamin B12 treated ND4 animals.

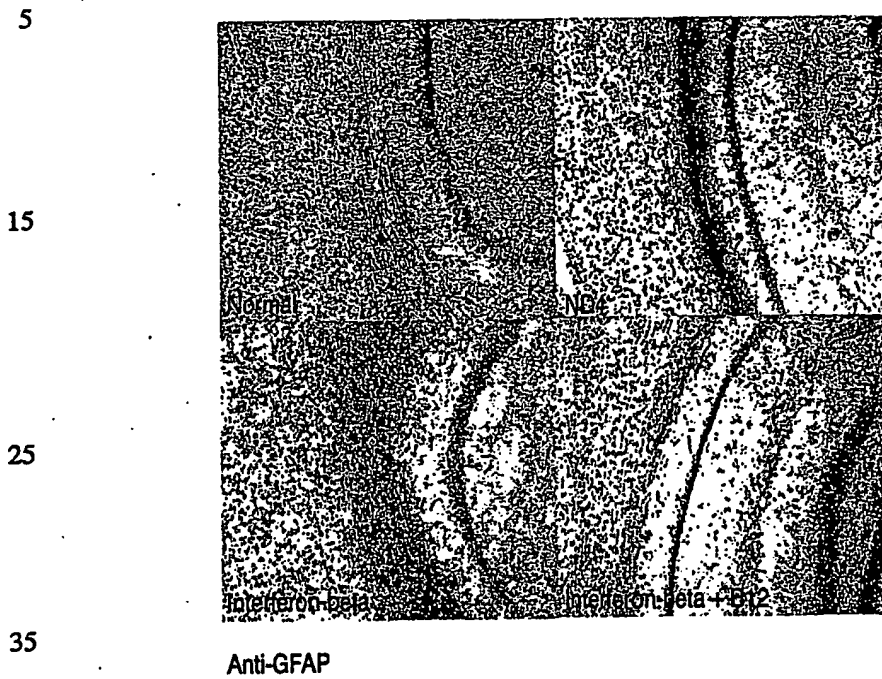
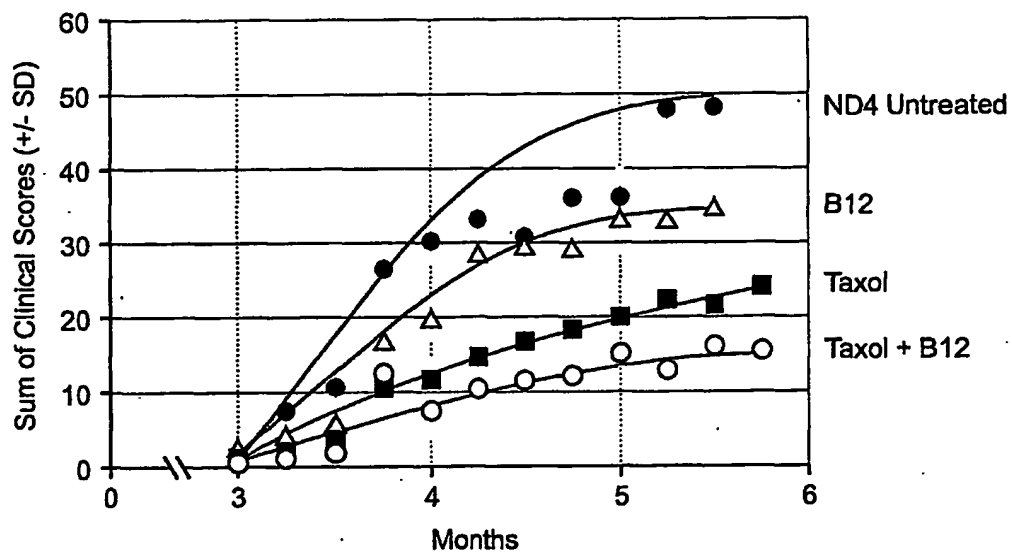


Figure 5. Effect of combination of vitamin B12 with paclitaxel (Taxol) in ND4 mouse model



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## INTERNATIONAL SEARCH REPORT

International Application No.

PCT/CA 02/00895

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 A61K38/21 A61K31/714 A61P35/00 A61P31/12 A61P29/00  
 //(A61K38/21,31:714)

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, PAJ, BIOSIS, EMBASE, SCISEARCH

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 01 28592 A (COLLINS DOUGLAS A ;MAYO FOUNDATION (US); UNIV MINNESOTA (US); HOGE) 26 April 2001 (2001-04-26) page 4, line 4 - line 8 page 11, line 10 -page 13, line 13 page 9, line 27 - line 33	1-14, 16-18, 20-24
X	US 5 574 018 A (HABBERFIELD ALAN D ET AL) 12 November 1996 (1996-11-12) column 23, line 9 - line 21; claim 5	1-11,14, 16-24
X	US 5 565 558 A (MCCULLY KILMER S) 15 October 1996 (1996-10-15) column 6, line 47 -column 7, line 22 column 12, line 31 - line 39; claim 2 -/--	1-11,14, 16-24

☒ Further documents are listed in the continuation of box C.☒ Patent family members are listed in annex.

## \* Special categories of cited documents:

\*A\* document defining the general state of the art which is not considered to be of particular relevance

\*E\* earlier document but published on or after the International filing date

\*L\* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

\*O\* document referring to an oral disclosure, use, exhibition or other means

\*P\* document published prior to the International filing date but later than the priority date claimed

\*T\* later document published after the International filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

\*X\* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

\*Y\* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

\*Z\* document member of the same patent family

Date of the actual completion of the international search

25 September 2002

Date of mailing of the international search report

11/10/2002

Name and mailing address of the ISA

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Authorized officer

Escolar Blasco, P

International Application No  
PCT/CA 02/00895

Form PCT/SA/210 (continuation of second sheet) (July 1982)

## INTERNATIONAL SEARCH REPORT

International Application No

PCT/CA 02/00895

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>KIRA J-I ET AL: "VITAMIN B12 METABOLISM AND MASSIVE-DOSE METHYL VITAMIN B12 THERAPY IN JAPANESE PATIENTS WITH MULTIPLE SCLEROSIS"</p> <p>INTERNAL MEDICINE, JAPANESE SOCIETY OF INTERNAL MEDICINE, TOKYO, JP, vol. 33, no. 2, 1 February 1994 (1994-02-01), pages 82-86, XP000650481 ISSN: 0918-2918 abstract</p> <p>-----</p>	15

# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/CA 02/00895

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:  
  
Although claims 18-23 are directed to a method of treatment of the human body, the search has been carried out and based on the alleged effects of the composition.
2. ☐ Claims Nos.:  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/CA 02/00895

Patent document cited in search report		Publication date	Patent family member(s)	Publication date
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US 5565558	A	15-10-1996	NONE	
US 6121249	A	19-09-2000	US 6323188 B1	27-11-2001